International Journal of Odonatology, 2016
Vol. 19, No. 4, 275–282, http://dx.doi.org/10.1080/13887890.2016.1259663

A description of the larva of *Mesopodagrion tibetanum australe* (Odonata: "Megapodagrionidae")

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(*Received 15 July 2016; final version received 8 November 2016*)

The larva of the genus *Mesopodagrion* was identified with the help of DNA barcoding and described for the first time. The larvae have flat horizontal gills resembling those of Argiolestidae but the adults lack setae on the shaft of the genital ligula. Molecular data are shown to be useful and necessary in larval identification and should be adopted as a standard tool in future studies.

Keywords: Zygoptera; Megapodagrionidae; *Mesopodagrion tibetanum australe*; larva; DNA barcoding; dragonfly

Introduction

Mesopodagrion McLachlan, 1896 is a small genus (only two extant species are known according to Schorr, Lindeboom, & Paulson, 2009), which mainly occurs in south China and Indo-China. Although a revised description of the adult has been available for several years (Yu & Bu, 2009) no larva of *Mesopodagrion* has been described to date. The larvae of megapodagrionids were divided into four groups by Kalkman, Choong, Orr, and Schütte (2010); the last group (Group 4), comprising species with flat horizontal gills and lacking setae on the shaft of the genital ligula, was treated as monophyletic. Kalkman et al. (2010), though lacking evidence, supposed that the larva of *Mesopodagrion* would fall into a group, referred to as Group 1, that have sack-like gills, and predicted that this genus together with two other Asian genera *Priscagrion* and *Sinocnemis*, "might have completely different and unexpected affinities". According to the phylogenetic study based on morphological characters, Yu and Bu (2011a) considered that *Mesopodagrion* should be a basal group of megapods. However, molecular phylogenetic work failed to recover a stable phylogenetic position of this genus (Dijkstra, Kalkman, Dow, Stokvis, & van Tol, 2014).

The larvae of relatively rare odonates such as *Mesopodagrion* are usually very difficult to find. Not only are individual numbers of natural populations often limited, but also the larvae may be difficult to rear to emergence, therefore not easy to identify. However, it is rare to encounter a larva just emerging, as was the case detailed in Yu and Bu (2011b). Linking larvae and adults by sympatry is sometimes dangerous (Wang & Yu, unpublished). Fortunately, the method of DNA barcoding provides a practical tool to compare larva and adult (Hebert, Penton, Burns, Janzen, & Hallwachs, 2004). Recently, with the help of DNA barcoding, the enigmatic larva of

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Mesopodagrion was discovered from Guizhou, China. The interesting characters of the larva will stimulate more discussions on the systematic position of this genus.

Material and methods

Samples

Larvae were found in a small montane swamp with a little brook running through it. The water was very shallow covered by weeds. Adults were present around the site in moderate numbers. Larvae were collected using a dip net with removal of litter by hand. Adults were collected with sweep net. Attempts were made to rear collected larvae in the laboratory in plastic containers but failed. All the living photos were taken in the field, before or just after collection, with a digital camera (Nikon D3200, Thailand). Character photos were taken in the laboratory using Zeiss V20 (Germany) microphotography system. Specimens were preserved in absolute ethanol and were examined and dissected under a Zeiss V8 stereomicroscope. In order to confirm the newly discovered larvae were the same species as the adults collected at the same habitat, four larval individuals and two adults were selected for the phylogenetic analysis. COI sequences of two *Philosina* and one *Mesopodagrion* (the unique one of this genus by now) from National Center for Biotechnology Information (NCBI) were also included in the analysis as outgroups. COI sequences from a total of nine specimens were used for molecular phylogenetic study (Table 1); of these sequences, six were obtained from samples that were collected in Guizhou, China at 2015. Samples were preserved in 90% ethanol during fieldwork, and subsequently stored in a freezer at –20°C in the laboratory after identification until DNA extraction.

DNA extraction and sequencing

Whole genomic DNA was isolated from muscle tissue of one or two legs from each sample using UniversalGen DNA Kit (Beijing ComWin Biotech, Beijing, China), following the manufacturer's instructions. Voucher specimens and genomic DNA were stored at –20°C. Primers designed by Yu, Xue, Hämäläinen, Liu, & Bu (2015) were used to amplify the target COI sequences, namely COIf1(5′-GRG CAT GRG CAG GWA TAG TNG-3′) and COIr1(5′-GGG TAG TCT GAR TAT CGT CGN GGT-3′). PCRs were carried out in 40 µl final reaction volumes containing 20 µl of 2 × Es Taq MasterMix (Beijing ComWin Biotech), 15.5 µl ddH_2O, 1.5 µl of each primer and genomic DNA. The thermal cycling program of the PCR procedure included an initial denaturation at 94°C for 2 min, followed by 33–35 cycles of 30 s at 94°C, 30 s at 51–52°C and 1 min at 72°C, ending with a final extension at 72°C for 8 min. All PCR

Table 1. Specimens used in the present study.

Species	Data source	ID/AN	Location	Date	Collector
Mesopodagrion tibetanum	NCBI	KF369442			
Philosina buchi	NCBI	KF369495			
Philosina alba	NCBI	KF369494			
M. t. austral	This study	MpLGSH01	Leigongshan, Guizhou, China	30 July 2015	Xin Yu
M. t. austral	This study	MpLGSH02	Leigongshan, Guizhou, China	30 July 2015	Xin Yu
M. t. austral	This study	MpLGSH03	Leigongshan, Guizhou, China	30 July 2015	Xin Yu
M. t. austral	This study	MpLGSH04	Leigongshan, Guizhou, China	30 July 2015	Xin Yu
M. t. austral	This study	MpLGSH05	Leigongshan, Guizhou, China	30 July 2015	Xin Yu
M. t. austral	This study	MpLGSH06	Leigongshan, Guizhou, China	30 July 2015	Xin Yu

products were visualized via 1% agarose gel electrophoresis and amplifications were purified using a gel extraction kit (Sangon Biotech, Shanghai, China), then sent to commercial companies (BGI TechSolutions, Shenzhen, China or GENEWIZ, Beijing, China) for sequencing based on Sanger's chain termination method. All fragments were sequenced in both directions.

DNA analysis

Sequences were edited and assembled in BioEdit v7.2.0 (Hall, 1999). Alignments of protein coding genes were translated to amino acids using MEGA v6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) to detect frameshift mutations and premature stop codons, which may indicate the presence of pseudogenes. Sequences were aligned using the ClustalX version 2.1 program package (http://www.clustal.org/) with default settings, and subsequently corrected manually in terms of the sequence chromatogram to ensure each mutation locus was credible. Phylogenetic analysis was performed using Bayesian inference with MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001). The GTR + I model of substitution, explored using MrModeltest v2.3 (Nylander, 2004), was used for the data matrix. All the acquired trees were set to 2 million generations and for every 1000 generations the chain was sampled. The Markov chain Monte Carlo (MCMC) process was run over four parallel chains, one cold and three incrementally heated. Generations with standard deviation values higher than 0.01 were burned. Trees were displayed and rendered with FigTree v1.4.0 (Rambaut & Drummond, 2007).

Result

The target COI sequences of all samples amplified and were sequenced successfully. The final dataset consisted of nine sequences, each 567 bp. *Mesopodagrion* in the analysis formed a good monophyletic group with strong support (Bayesian posterior probability, BPP = 1).

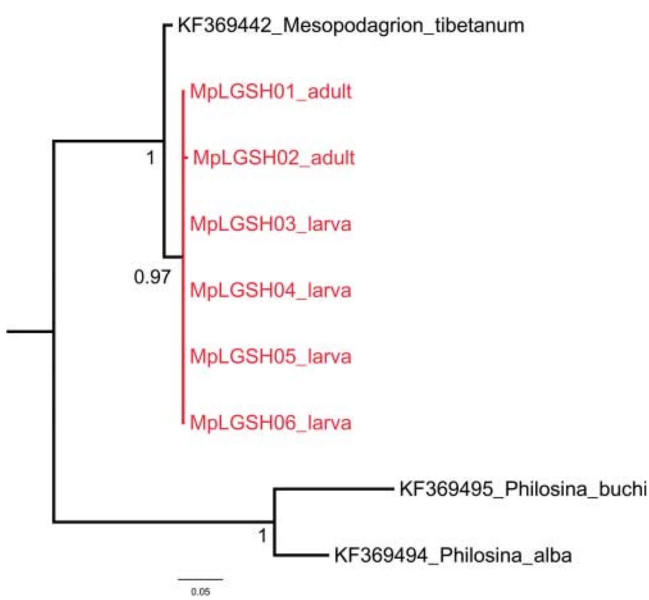

Figure 1. Phylogenetic reconstruction based on Bayesian inference with COI (567 bp). Bayesian posterior probabilities are indicated at nodes and adults and larvae of Guizhou specimens are in red colour.

Mesopodagrion tibetanum (sequence from NCBI, KF369442) was recovered as sister group to all specimens from Guizhou (Figure 1). The six Guizhou individuals (including two adults and four larvae) formed a clade with no further divided branches also with strong support (BPP = 0.97), which implied they are the same species. According to Yu and Bu (2009) the adults were identified as *Mesopodagrion tibetanum austral*.

Taxonomic account

Larva of *Mesopodagrion tibetanum austral* Yu & Bu, 2009 (Figure 2a).

Material examined

2♂, Leigongshan, Guizhou, China, 30 July 2015, Xin Yu leg.; 4 larvae (ca F-3), ditto.

Diagnosis

A robust zygopteran with a large head, short legs and broad fan-like horizontal gills. Ground colour of body brown, lacking distinct bands or marks (Figure 2).

Description

Head: Relatively broad, exceeding maximum width of mesothorax; in dorsal view general shape a compressed pentagon, with the occipital margin deeply excavated to form a rounded margin.

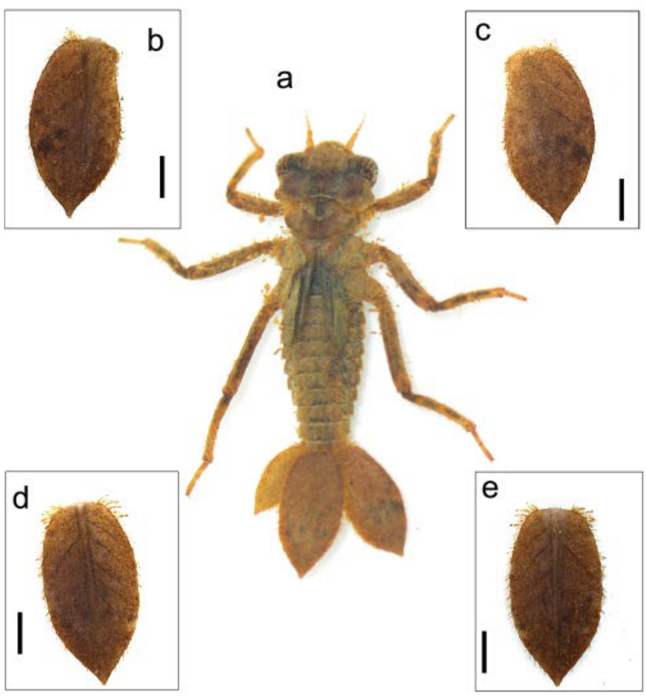

Figure 2. (a) Larval habitus of *Mesopodagrion tibetanum austral*; (b) right lateral caudal gill, ventral view; (c) same, dorsal view; (d) middle caudal gill, dorsal view; (e) same, ventral view. Scale bars = 1 mm.

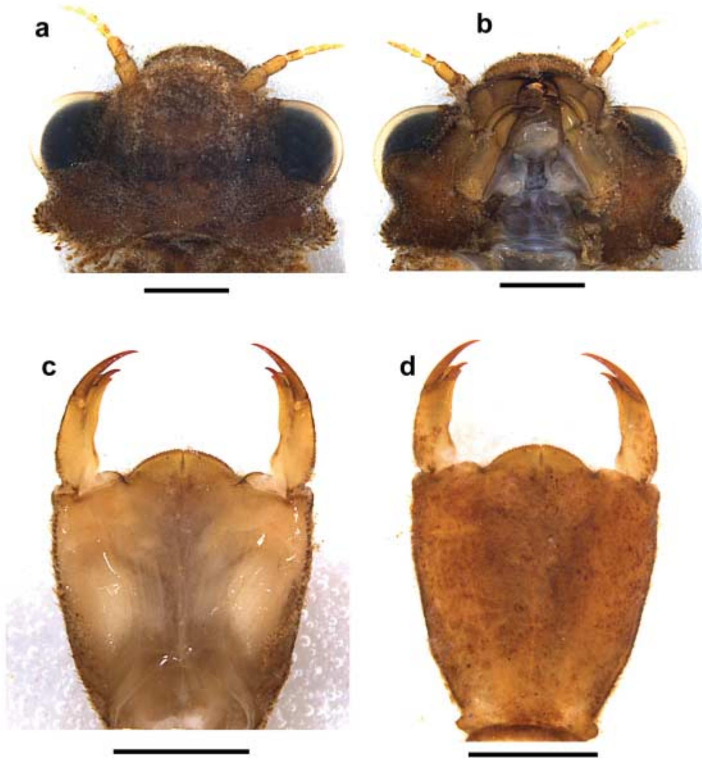

Figure 3. (a) Head of the larva, dorsal view; (b) same, ventral view, mask removed; (c) prementum and labial palps, dorsal view; (d) same, ventral view. Scale bars = 1 mm.

Cephalic lobes swollen to form a pair of large protuberances covered with dense strong spines and fan-shaped setae (Figure 3a, b). Antennae 7-segmented, relatively short and evenly tapered, segments from base to apical become shorter gradually with segment 7 just one third length of segment 1 (Figure 2a). Labium (Figure 3c, d) trapezoidal, not elongate; prementum just as long as broad and basally narrow. Ligula convex with uniform marginal denticles; median cleft short. Labial palp (Figure 3c, d) robust, lacking setae; distally with two strong, short, incurved teeth and small short process on the inner margin just basad of inner tooth; movable hook long, incurved and robust. Maxilla almost as long as broad. Galea and lacinia partly fused; lacinia terminating in four long sharp spines, forming a curved, inward-directed, pitchfork-like structure; galea with three shorter spines directed upward. Palp with short basal segment and a single long banana-shaped terminal segment, reaching to half of most distal spines on galeo-lacinia, covered in dense long setae for the distal two-thirds of its length (Figure 4e–h). Right mandible (Figure 4c, d) with four well-developed incisors and a fifth innermost tooth; outermost (ventral) tooth with small secondary tooth well before its apex; molar crest produced to form a well-defined curved bifid spine (thus, in the terminology of Watson [1956], R $1'1234$ y ab, $2 < 1 < 3 < 4$). Left mandible (Figure 4a, b) with similar incisors; molar crest produced straight, with distal edge serrated with 5 fine cusps (L $1'1234$ 0 a($m^{1,2,3}$)b, $2 < 1 < 3 < 4$, $a > b$, $m^1 > m^2 > m^3$).

Thorax Prothorax robust, almost elliptical; Meso- and metathorax irregular, rounded, sub-rectangular shape. Legs moderately short and strong, bearing scattered short thorns and hairs, lacking bands or other marks; legs progressively slightly longer from pro- to metathorax. Wing pads moderate long, divergent, flat and narrow, almost reaching to middle of S4.

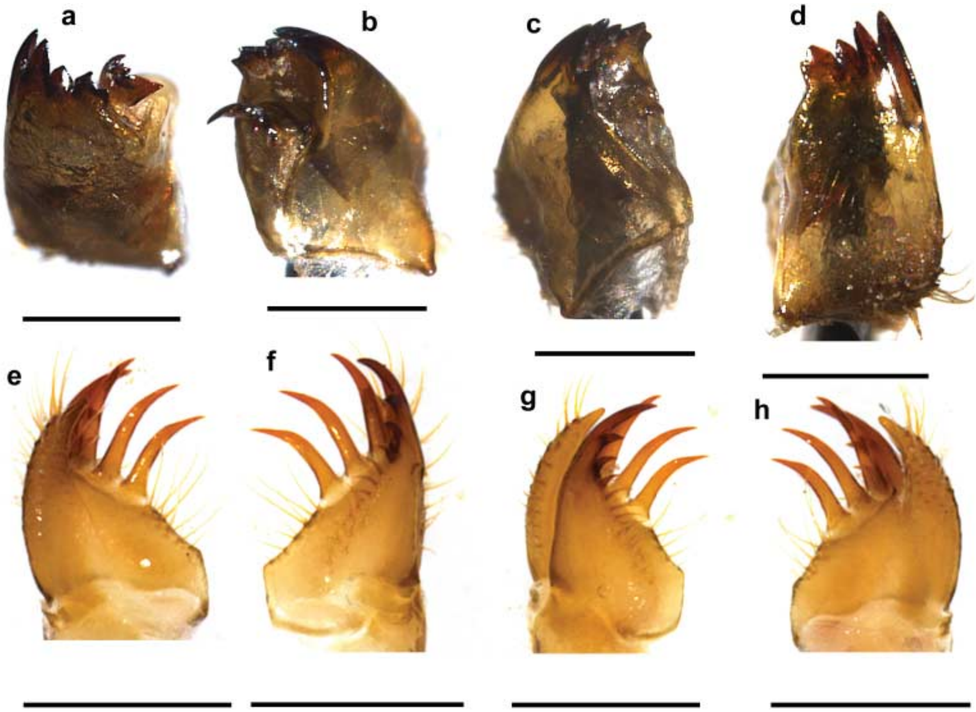

Figure 4. (a) Left mandible, posterior surface; (b) same, inner surface; (c) right mandible, inner surface; (d) same, posterior surface; (e) left maxilla, posterior surface; (f) same, inner surface; (g) right maxilla, inner surface; (h) same, posterior surface. Scale bars = 0.5 mm.

Figure 5. Habitat of the *Mesopodagrion tibetanum austral* from Leigongshan, Guizhou. Habitus of adult shows in the inner panel.

Abdomen Robust and elongate moderately, with strong long setae on the posterior edge of each segment. No spines or dorsal protuberances present. Gonapophyses not developed yet. Quite large, flat, fan-like horizontal gills covered with moderate dense short setae (Figure 2b–e); overall colour rather dark without any pattern.

Measurements (mm). Body length (including caudal gills) 15.9–16.6.

Microhabitat and behaviour

Both larvae and adults were found in a shallow montane swamp surrounding a little brook (Figure 5). The swamp was covered by dense marsh weeds. No high trees provided shade and sunlight shone down directly. Larvae were found, either concealed amongst fallen submerged or semi-submerged leaves and sticks, or on bottom mud in shallow water, usually inactive. The body of larvae were often covered by mud or fungi making them very difficult to detect from their background.

Discussion

Larvae of *M. t. australe* possess flat horizontal gills which strongly resemble those of *Podolestes* (Choong & Orr, 2010) and *Trineuragrion* (Marinov, 2012). Therefore, *Mesopodagrion* may belong to Argiolestidae if the adults also lack setae on the shaft of the genital ligula (Dijkstra et al., 2014; Kalkman et al., 2010). As Kalkman et al. (2010) have indicated, the adults of *Mesopodagrion* do indeed have the setae, which I have confirmed on all my specimens including most of the specimens studied in Yu and Bu (2009) and all the newly collected Guizhou specimens. Should the presence of setae just be treated as an exceptional case? If so, *Mesopodagrion* will represent the northernmost distribution of Argiolestidae (Kalkman & Theischinger, 2013). However, it is too early to draw a conclusion since more robust phylogenetic work is needed to confirm this.

This study has shown that molecular methods, such as DNA barcoding, are helpful tools to tackle the difficult job of odonate larva identification. In some cases, when several closely related genera or species occur sympatrically, and their larvae are unknown, linking larvae and adults together empirically may be questionable. Molecular proofs for future studies on larvae identification will be inevitable, even for some species for which the larvae have already been described.

Acknowledgements

I would like to thank Dr Junli Xue for helping with the molecular work. I am grateful to the two reviewers and the editor for their valuable remarks and suggestions. This project was supported by the National Natural Science Foundation of China (No. 31572299) and the grant of Ministry of Science and Technology of China (No. 2015FY210300).

References

Choong, Ch.-Y., & Orr, A. G. (2010). The larva of *Podolestes orientalis* from West Malaysia, with notes on its habitat and biology (Odonata: Megapodagrionidae). *International Journal of Odonatology*, *13*, 109–117. doi:10.1080/13887890.2010.9748365

Dijkstra, K.-D. B., Kalkman, V. J., Dow, R. A., Stokvis, F. R., & van Tol, J. (2014). Redefining the damselfly families: the first comprehensive molecular phylogeny of Zygoptera (Odonata). *Systematic Entomology*, *39*, 68–96. doi:10.1111/syen.12035

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, *41*, 95–98. doi:10.1021/bk-1999-0734.ch008

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *PNAS*, *101*(41), 14812–14817. doi:10.1073/pnas.0406166101

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*(8), 754–755. doi:10.1093/bioinformatics/17.8.754

Kalkman, V. J., Choong, Ch.-Y., Orr, A. G., & Schütte, K. (2010). Remarks on the taxonomy of Megapodagrionidae with emphasis on the larval gills (Odonata). *International Journal of Odonatology*, *13*, 119–135. doi:10.1080/13887890.2010.9748366

Kalkman, V. J., & Theischinger, G. (2013). Generic revision of Argiolestidae (Odonata), with four new genera. *International Journal of Odonatology*, *16*, 1–52. doi:10.1080/13887890.2012.749450

Marinov, M. G. (2012). Description of the larva of *Trineuragrion percostale* Ris (Odonata: Megapodagrionidae) with a key to the larvae of New Caledonian genera of Megapodagrionidae. *International Journal of Odonatology*, *15*, 241–248. doi:10.1080/13887890.2012.718234

Nylander, J. A. A. (2004). *MrModeltest v2*. Program distributed by the author, Evolutionary Biology Centre, Uppsala University, Uppsala.

Rambaut, A., & Drummond, A. J. (2007). *Tracer v1.5*. Retrieved from http://beast.bio.ed.ac.uk/Tracer.

Schorr, M., Lindeboom, M., & Paulson, D. R. (2009). List of Odonata of the world (Part 1, Zygoptera and Anisozygoptera) [Online]; Retrieved from http://www.pugetsound.edu/academics/academic-resources/slater-museum/biodiversity-resources/dragonflies/world-odonata-list2, accessed July 27, 2016.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. doi:10.1093/molbev/mst197

Watson, M. C. (1956). The utilization of mandibular armature in taxonomic studies of anisopterous nymphs. *Transactions of American Entomological Society*, *81*, 155–209.

Yu, X., & Bu, W. J. (2009). A revision of *Mesopodagrion* McLachlan, 1896 (Odonata: Zygoptera: Megapodagrionidae). *Zootaxa*, *2202*, 59–68. doi:10.15468/wrm0pr

Yu, X., & Bu, W. J. (2011a). A preliminary phylogenetic study of Megapodagrionidae with focus on the Chinese genera *Sinocnemis* Wilson & Zhou and *Priscagrion* Zhou & Wilson (Odonata: Zygoptera). *Hydrobiologia*, *665*, 195–203. doi:10.1007/s10750-011-0622-7

Yu, X., & Bu, W. J. (2011b). A description of the larva of *Pseudolestes mirabilis* Kirby (Odonata: Pseudolestidae). *International Journal of Odonatology*, *14*, 105–110. doi:10.1080/13887890.2011.592486

Yu, X., Xue, J. L., Hämäläinen, M., Liu, Y., & Bu, W. J. (2015). A revised classification of the genus Matrona Selys, 1853 using molecular and morphological methods (Odonata: Calopterygidae). *Zoological Journal of the Linnean Society*, *174*, 473–486. doi:10.1111/zoj.12253